

REMARKS

This is a full and timely response to the Office Action mailed January 13, 2005, submitted concurrently with a three month extension of time to extend the due date for response to July 13, 2005.

No claims have been amended in this response. Thus, claims 20-32 are currently pending in this application.

In view of this response, Applicants believe that all pending claims are in condition for allowance. Reexamination and reconsideration in light of the above amendments and the following remarks is respectfully requested.

Rejection under 35 U.S.C. §112

Claim 26 is rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. Applicant respectfully traverses this rejection.

Based on the Examiner's remarks in the action, it appears that the Examiner is unclear as to how the Applicant obtained the 1:25 ratio from the disclosure of "*400 micrograms DNA/ml of 10 mg/ml*" in the specification (see page 10, lines 10-16, of the specification). For the Examiner's information, the term "*10 mg/ml*" refers to the amount of lipid and thus *400 micrograms DNA/ml of 10 mg/ml of lipid* is equivalent to a DNA:lipid ratio of 1:25 (i.e. 10 mg of lipid per .4 mg of DNA). Hence, it is clear that the concentration by weight of liposome being 25 times the concentration by weight of plasmid is supported by the teachings in the specification.

Thus, withdrawal of this rejection is respectfully requested.

Rejections under 35 U.S.C. §102 and §103

Claims 20-23 and 26 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Sha et al. Further, claims 20, 24, 25 and 27-32 are rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Sha et al. and Promega Catalog. Applicant respectfully traverses these rejections.

To constitute anticipation of the claimed invention, a single prior art reference must teach each and every limitation of the claims. Further, to establish a *prima facie* case of obviousness, the cited references, in combination, must teach or suggest the invention as a whole,

including all the limitations of the claims. Here, in this case, Sha et al. fails to teach a liposomal vaccine composition comprising a plasmid **encapsulated within a liposome**.

Despite our arguments of June 22, 2004, the Examiner has maintained these rejections since it is unclear from the teachings of Xu et al. and Bajoghli whether the DNA in Sha et al. are complexed or encapsulated within the liposomes. To clarify the Examiner's understanding in this regard, Applicant has provided three additional references which clearly and unequivocally demonstrate that DNA mixed with DOSPER (cationic lipid) liposomes (as taught by Sha et al. reference), is clearly complexed with liposomes, and not encapsulated in liposomes. The three references are:

- 1) Lampela, P. Elomaa, M., Ruponen, M. Urtti, A., Mannisto, P.T. and Raasmaja, A. Different synergistic roles of small polyethylenimine and Dosper in gene delivery. *J. Controlled Release* 88:173-183, 2003.
- 2) Yerushalmi, N., Brinkmann, U., Brinkman, E., Pai, L. and Pastan, I. Attenuating the growth of tumors by intratumoral administration of DNA encoding *Pseudomonas* exotoxin via cationic liposomes. *Cancer Gene Therapy* 7:91-96, 2000.
- 3) Kott, M., Haberland A. Zaitsev, S., Buchberger, B., Morano, I., and Bottger, M.A. new efficient method for transfection of neonatal cardiomyocytes using histone H1 in combination DOSPER liposomal transfection reagent. *Somatic Cell and Molecular Genetics* 24:257-261, 1998.

In Lampela et al., the reference clearly states that DOSPER and DNA (plasmid) formed globular complexes of various sizes having striped fine structures (see page 178, section 3.5, lines 7-9, of Lampela et al.). Using transmission electron microscopy (TEM), Lampela et al. show TEM images (see page 179, Fig 4) of the DOSPER liposomes (no complex, panels A1 and A2) and DOSPER + DNA (complexes, panels E1 and E2). These electric micrographs clearly show that DOSPER and plasmid DNA form globular complexes, rather than DNA being encapsulated in liposomes (no complex formation). Furthermore, the reference clearly describes in the Abstract DNA-DOSPER compositions as DOSPER-DNA complexes (see Abstract, lines 6, 7 and 8, of Lampela et al.).

In Yerushaima et al., DOSPER liposomes are used to deliver plasmid DNA encoding *Pseudomonas* exotoxin. In the reference, it is explicitly described that:

*"[I]n this study, we chose to use cationic lipids with a multivalent cationic lipid head group (spermine), dioctodeylamidoglycyl spermine (DOGS), and 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl-amid (DOSPER). **The cationic liposomes form COMPLEX with DNA through charge interactions**, and the net positively charged **COMPLEX** can interact*

with cells to produce successful transfections” (see page 91, paragraph 3, lines 8-15, of Yerushaima et al.).

In Kott et al., the reference describes “[B]riefly, for complexation, 2 µg plasmid DNA... were mixed with varying amounts of the following transfection reagents: DOTAP or DOSPER” (see page 258, paragraph 6, lines 14-19, of Kott et al.). Furthermore, the reference also states that “[I]n this study, we investigated possibilities to improve the transfection efficiency of commercial lipid formulations (lipofection) on cultured CM by interacting histone H1-complexed DNA with cationic lipid. As an exemplary lipid reagent we used the polycationic reagent DOSPER carrying positively charged amino groups.” (see page 259, paragraph 3, line 3-10, of Kott et al.).

Like Lampela et al., Yerushaima et al., and Kott et al., Sha et al. describes mixing the influenza HA gene with Dosper liposome (see page 22 of Sha et al., “20µg of pjw4303 DNA was mixed with 40µg of Dosper in HBS”), and discusses that cationic liposomes have been shown to efficiently complex with DNA (see page 26 of Sha et al.). In other words, in view of the enclosed references, Sha et al. clearly describes experiments which were conducted to determine the effectiveness of DNA/lipid complexes and not composition of DNA encapsulated within liposomes.

In contrast to Sha et al., the present invention requires that the plasmid DNA be encapsulated within a liposome. In order to clearly show that the claimed vaccine composition comprises a plasmid encapsulated within a liposome, Applicant has enclosed experiment data from Applicant’s particle size measurements of the liposome-encapsulated DNA vaccine as described in present specification. The liposome-encapsulated DNA particle size distribution was measured using a Malvern Zetasizer instrument. The experimental data clearly shows the liposome-encapsulated HA DNA are homogenous in size (about 100 nm), rather than bigger complexes with different or heterogenous size distributions (larger than 1µM in diameter).

Furthermore, as stated previously, the fact that Sha et al. used plasmid DNA-DOSPER liposome complexes to induce antiviral immunity in the respiratory tract, was shown to be not effective in protecting mice against influenza virus challenge (see results, page 26, lines 1-6, last paragraph, of Sha et al.). This is in contrast to the present invention which uses liposome-encapsulated plasmid DNA to protect mice against lethal challenge with influenza virus infection. Applicant’s present patent application provides clear scientific evidence that liposome-encapsulated DNA vaccine provided 100% protection to mice against influenza virus

challenge (see page 14, lines 5-14, and Figures 3 and 4 of the present specification). These completely different results show that the liposomal vaccine composition of the present invention (i.e. *plasmid encapsulated within a liposome*) is fundamentally distinct in terms of structural makeup, biological activity and function from the DNA/liposome complex of Sha et al. These superior results also suggest that encapsulation of plasmid DNA within liposomes (rather than complexing with liposomes) is important in providing protection. As the Examiner already knows, a showing of superior and unexpected properties can rebut a *prima facie* case of obviousness. *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963).

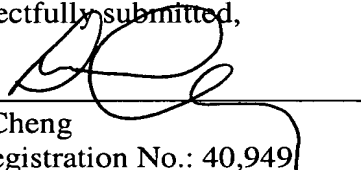
Thus, for these reasons, withdrawal of this rejection is respectfully requested.

CONCLUSION

For the foregoing reasons, all the claims now pending in the present application are believed to be clearly patentable over the outstanding rejections. Accordingly, favorable reconsideration of the claims in light of the above remarks is courteously solicited. If the Examiner has any comments or suggestions that could place this application in even better form, the Examiner is requested to telephone the undersigned attorney at the below-listed number.

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Respectfully submitted,

By 
Lee Cheng
Registration No.: 40,949

RADER, FISHMAN & GRAUER PLLC.
1233 20th Street, N.W.
Suite 501
Washington, DC 20036
(202) 955-3750
Attorney for Applicant

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Different synergistic roles of small polyethylenimine and Dosper in gene delivery

Pasi Lampela^a, Matti Elomaa^b, Marika Ruponen^b, Arto Urtti^b, Pekka T. Männistö^a,
Atso Raasmaja^{a,*}

^aDepartment of Pharmacology and Toxicology, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland

^bDepartment of Pharmaceutics, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland

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Abstract

Low-molecular-weight PEIs and cationic liposomes can be combined resulting in a synergistic increase in transfection efficiency as we have reported earlier. Here, we have further investigated the potential mechanisms of this synergy. Complex morphology, complex sizes and DNA condensation were studied using transmission electron microscopy, light scattering methods and ethidium bromide exclusion, respectively. Cellular uptake, transfection efficiency, and effect of proton pump inhibitor bafilomycin A1 were examined in cell cultures. The cellular uptake of DNA was negligible with PEI2K–DNA complexes, whereas the uptake of the PEI2K–DNA–Dosper or the Dosper–DNA complexes was maximally about 40%. The number of transfected cells was two times higher with PEI2K–DNA–Dosper complexes than with Dosper–DNA complexes. The PEI2K–DNA–Dosper combination was slightly less sensitive to bafilomycin A1 than the PEI25K–DNA or Dosper–DNA complexes. There were no differences between PEI2K and PEI25K in DNA condensation. Dosper condensed DNA slightly more in PEI2K complexes. The PEI25K–DNA complexes were much smaller (<250 nm) than the PEI2K–DNA complexes (0.5–12 μ m) which were also rather polydisperse. It is suggested that two independent mechanisms would lead to synergistic transfection efficiency: (1) Dosper improves the cellular uptake of PEI2K–DNA complexes, and (2) PEI2K improves a transfer of the complexes from lysosomes to nucleus.

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1. Introduction

In gene therapy, viral vectors and plasmid DNA are used to generate new ways to treat diseases. Since plasmid DNA enters the cells poorly, different transfection reagents such as cationic lipids (for

review, see Ref. [1]) and cationic polymers [2–4] have been developed to shuttle plasmid DNA into the cells. For successful transfection, DNA must pass the cell membrane and find its way into the nucleus.

Cationic lipids are widely studied as gene transfection reagents (for review, see Ref. [5]). They form liposomes or micelles that bind electrostatically to negatively charged DNA forming complexes. These complexes enter the cells via endocytosis [6,7]. After endocytosis the plasmid DNA must escape from the

*Corresponding author. Tel.: +358-17-162-407; fax: +358-17-162-424.

E-mail address: atso.raasmaja@uku.fi (A. Raasmaja).

endosomes into the cytoplasm. Xu and Szoka [8] proposed that DNA–liposome complexes destabilize the endosomal membrane resulting in flip–flop of anionic lipids from the endosomal membrane. Anionic and cationic lipids form neutral ion pairs displacing the plasmid DNA from the complex and permitting its entry into the cytoplasm. Plasmid DNA must also be released from liposome–DNA complex before entering the nucleus [9].

PEIs are branched or linear cationic polymers, that have been widely used for transfection studies (for review, see Ref. [10]). PEI–DNA complexes are taken into the cells via endocytosis. Since every third atom of PEI molecule is a nitrogen that can be protonated at endosomal pH range [3], PEI has the ability to capture protons that are pumped into endolysosomes. This is, presumably, followed by passive chloride influx into the endosomes and subsequent osmotic swelling and disruption of endosomes. This permits the escape of endocytosed DNA complexes [3,11]. Recently, Merdan et al. [12] showed that the release of PEI/RNA complexes from endolysosomes occurs as a sudden event. PEIs are shown to induce local lysosomal membrane damages, which allows the PEI–DNA complex to enter the cytoplasm [13]. PEIs are also shown to direct DNA into the nucleus [14]. In general, the PEIs tend to have wide distribution of molecular weights [15]. PEIs with mean MW > 11 900 have shown significant transfection efficiency, PEI25K (mean MW 25 000) being the most widely used molecular size [11–14,16–18]. Combination of large and small PEIs is found to increase transfection efficiency [19]. Also, when PEI25K was fractionated in different pools, the pool containing PEIs with molecular weight of 0.5–10 kDa was found efficient in gene transfection [15]. PEIs with mean molecular weights ≤ 2000 are inactive as transfection agents as such, but they can be used in combinations [20,21].

Previously, we have shown [21] a synergism between cationic lipid Dosper and small molecular weight PEI in transfection. In the present study, we have investigated the mechanism of the synergy using physicochemical (complex morphology, size, DNA condensation) and biological methods (cell uptake and transfection, and the effect of proton pump inhibitor bafilomycin A1).

2. Materials and methods

2.1. Chemicals

Polyethylenimines, ONPG (*o*-nitrophenol- β -D-galactopyranoside) and bafilomycin A1 were purchased from Sigma-Aldrich (USA). Dosper and Dotap were from Boehringer Mannheim (Germany). Dulbecco's modified Eagle's Medium, fetal bovine serum and penicillin–streptomycin were from Life Technologies (UK).

2.2. Plasmids

BPVTKlacZ plasmid coding β -galactosidase was synthesized by Dr Mart Ustav [22]. In cellular uptake and GFP expression experiments, CMV-driven pCR3 plasmid coding GFP (green fluorescence protein) plasmid and EMA (ethidium monoazide)-labeled CMV–GFP plasmid described in Ruponen et al. [23] were used.

2.3. Plasmid labeling

Plasmid–DNA was covalently labeled with EMA according to the procedure described earlier [23]. Briefly, EMA in water (5 μ g/ml) was added to the CMV–GFP plasmid in water (200 μ g/ml). After 10 min incubation at room temperature the mixture was exposed to the UV light at 312 nm for 2 min. Free EMA label was removed by gel filtration on NAP-10 columns (Amersham Pharmacia Biotech, UK). Intercalated EMA label was removed by extractions with CsCl-saturated isopropanol after adding of cesium chloride to the labeled DNA at the concentration of 1.1 g/ml. Cesium chloride was removed by dialysis, and the labeled DNA was recovered by ethanol precipitation.

2.4. Cell culture

The RAA SMC cell line (smooth muscle cells from rabbit aortic media) was a kind gift from Dr Seppo Ylä-Herttua (University of Kuopio, Finland). The cells were grown in Dulbecco's modified Eagle medium supplemented with 9% heat-inacti-

vated fetal bovine serum and penicillin (90 U/ml)–streptomycin (90 µg/ml) at 37 °C and 5% CO₂.

2.5. Transfection protocol

Subconfluent SMC (rabbit aortic smooth muscle) cells were transfected as described earlier [21]. Briefly, complexes were prepared by mixing 1 µg (for 24-well plates) or 2.5 µg (for 6-well plates) DNA (1 µg DNA/30 µl 150 mM NaCl–20 mM Hepes) with PEI-solution (in equal volume with DNA), and Dosper (diluted 1:4 in NaCl–Hepes, Dosper/DNA ratios varying between 0–10 depending on the experiment) was then added to DNA–PEI complexes. These complexes were then pipetted to wells containing 1 ml (24-well plates) or 2 ml (6-well plates) serum-free DMEM. Transfection time was 6 h, after which cells were harvested in cellular uptake experiments for measurements of EMA-labeled DNA. In experiments done with GFP or *lacZ* reporter gene, 1 or 2 ml of growth medium containing serum and penicillin–streptomycin was changed to wells and cells were incubated for 42 h. PEI–DNA ratios are expressed as PEI amine nitrogen/DNA phosphate ratio (N/P), and Dosper–DNA ratios as Dosper (µg)/DNA (µg) (w/w).

In Dotap-mediated transfection, transfection complexes were prepared by mixing 1 µg DNA (1 µg DNA/30 µl NaCl–Hepes) with 7.5 µg Dotap (diluted 3:7 in NaCl–Hepes), and transfection was performed as above.

2.6. Plasmid uptake and expression (EMA, GFP)

EMA-labeled DNA was used as marker for intracellular delivery of DNA, and cells were collected after transfection of 6 h. Cells transfected with unlabeled pCMV GFP were incubated for 42 h before the cells were collected. Before collection, the cells transfected with EMA-labeled DNA were washed with PBS, and incubated with 1 ml of 1 M NaCl for 30 s to remove complexes bound to cell surface. Then, the cells were washed twice with PBS and harvested. Both the EMA- and GFP-transfected cells were harvested from the wells with 0.7 ml trypsin (0.25%)–EDTA (0.02%). Then, the cells were fixed by incubating them for 10 min in 1%

paraformaldehyde, washed twice with 1% paraformaldehyde and stored in dark at +4 °C prior to their analysis by flow cytometry.

DNA uptake and transgene expression were analysed by flow cytometry (FACScan, Becton Dickinson) as described earlier [23]. Argon ion laser (488 nm) was the excitation source and fluorescence for GFP was detected at 525 nm (FL1) and for EMA (FL3) at 670 nm. 10 000 events were collected for each sample. The number of GFP and EMA positive cells were analyzed from the FL1 versus FL3 dot plot by setting a gate according to control.

2.7. Effect of bafilomycin A1

Subconfluent SMC cells were transfected with BPVTKlacZ plasmid. After changing the growth medium to serum-free transfection medium, endosome proton pump inhibitor bafilomycin A1 (specific inhibitor of vacuolar type H⁺-ATPase) diluted in DMSO or plain DMSO was pipetted into wells. After 10 min incubation, DNA complexes were pipetted into the wells as described above. After 6 h transfection, 1 ml of growth medium was changed into the wells, and the cells were further incubated for 42 h. Then, the cells were washed with PBS and they were lysed with 150 µl lysis reagent (25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF (phenylmethyl sulfonyl fluoride)), and centrifuged at 13 000 rpm for 5 min (Eppendorf Centrifuge 5415C, Eppendorf-Netheler-Hinz, Germany). The activity of β-galactosidase was measured with an ONPG assay from the supernatant: 1–10 µl of the supernatant, up to 100 µl of water and 100 µl of ONPG solution (2 mM MgCl₂, 1 mM β-mercaptoethanol, 1.33 mg/ml ONPG (*o*-nitrophenol-β-D-galactose) in 0.2 M sodium phosphate buffer) were placed in a 96-well plate and incubated up to 1 h at room temperature. The reaction was stopped with 1 M Na₂CO₃, and samples were analysed by measuring absorbance at 405 nm with the Bio-Tek Elx-800 microplate reader (Bio-Tek Instruments, USA) and KC-3 PC-program.

2.8. DNA condensation

Assays were carried out according to Ref. [18].

Briefly, the assays were carried out in 96-well plates in NaCl–Hepes buffer, pH 7.4. DNA (0.6 μg) and ethidium bromide (4 $\mu\text{g}/\text{ml}$) was added into the wells. Ethidium bromide binds to DNA and emits the maximum fluorescence signal. Then, different amounts of PEIs were added to wells and fluorescence was read after 10 min incubation. Then, different amounts of Dosper was added and fluorescence was read again after 10 min incubation. Decrease of fluorescence results in condensation of DNA. Experiments were performed using Bio-Tek FL500 microplate fluorescence reader (Bio-Tek Instruments, USA) at 530 nm excitation and 590 nm emission wavelengths.

2.9. Particle sizing

Complex sizes were determined by dynamic light scattering using Nicomp 380 ZLS zeta potential/particle sizer (Particle Sizing Systems, Santa Barbara, CA, USA). DNA–PEI complexes were made by adding PEI in 105 μl of NaCl–Hepes into 105 μl of NaCl–Hepes containing 3.5 μg of DNA. After 10 min incubation, particle sizes were measured for 5 min and immediately thereafter for another 5 min. After this, Dosper was added to PEI–DNA complexes, incubated for 10 min, and particle sizes were again measured with Nicomp.

2.10. TEM

The complexes were studied under transmission electron microscope (TEM) (JEM 1200 EX, Jeol, Japan). In the negative staining by uranyl acetate the sample is precipitated on carbon coated surface and fixed with the dye [24]. The complexes were prepared as described in Section 2.5 above. Ten μl of sample was added on copper grid, having fresh carbon coating, for 2 min. Then buffer was removed carefully by the edge of a moist filter paper and the grid was negatively stained with a droplet of 2% uranyl acetate (aqueous solution, pH 4.2) for 2 min. After removal of the stain droplet the grid was air-dried. The transmission electron microscopy was performed using voltage of 80 kV and magnifications of 30 000 and 100 000. Experiments were repeated four times. Negative controls were H_2O , NaCl–Hepes and different reagents (DNA, Dosper and

PEIs in NaCl–Hepes). The structures found with controls were evaluated as artifacts, and they were not included in the figures with transfection complexes.

2.11. Statistics

Unpaired Student's *t*-test was used to analyse the cellular uptake and expression of plasmids.

3. Results

3.1. Cellular uptake and plasmid expression

EMA-labeled plasmid and GFP plasmid were used to find out how many cells take up DNA and express transgene, respectively. We selected N/P ratios 8 for PEI25K and 2.5 for PEI2K for this experiment based on our previous work [21]. The results are presented in Fig. 1. The highest cellular uptake ($70.4 \pm 3.4\%$) was achieved with PEI25K (N/P=8) (Fig. 1A). With plain PEI2K (N/P=2.5), the cellular uptake was less than 2%. However, when a low ratio of Dosper (Dosper/DNA=1) was added to PEI2K–DNA complex, cellular uptake increased to $27.7 \pm 3.1\%$. The best cellular uptake for both Dosper alone and different PEI–2K–Dosper combinations was approximately 40%.

The highest expression was achieved with PEI2K–Dosper 7.5 combination ($14.8 \pm 0.5\%$). When Dosper was used alone in transfection, the highest level was $7.4 \pm 2.0\%$ (Dosper 7.5). However, only $3.0 \pm 0.5\%$ of cells transfected with the reference reagent, PEI25K, were expressing GFP (Fig. 1B).

Ratio of GFP-expressing cells/EMA-positive cells indicates that at optimal amount of Dosper (7.5) in PEI2K–Dosper mediated transfection, 33% of the cells that took up DNA complexes started to produce transgene protein (Table 1). Importantly, the GFP/EMA ratio at each Dosper concentration was always higher with PEI2K–Dosper–DNA than with Dosper–DNA complexes. With plain PEI2K, both cellular uptake (0.545%) and GFP expression (0.025%) were minimal (Fig. 1 and Table 1).

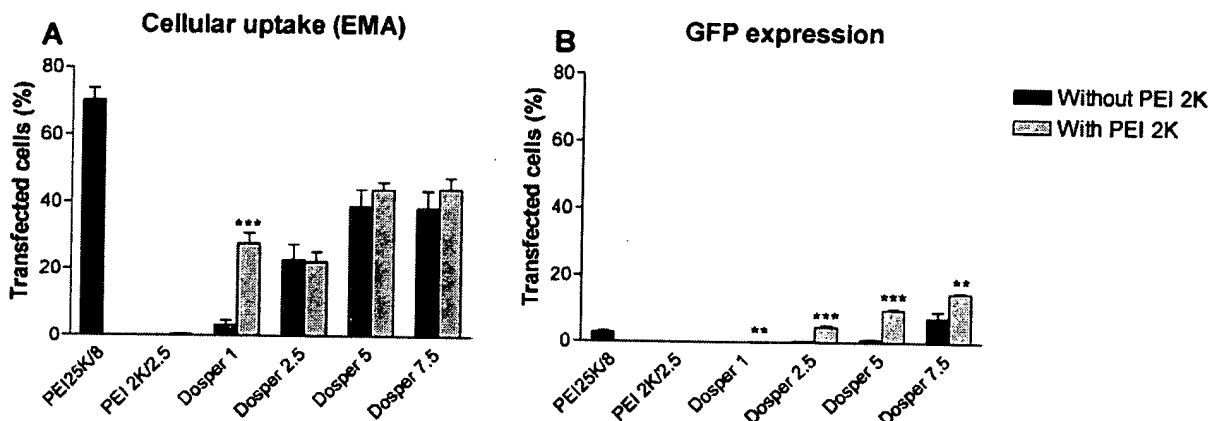


Fig. 1. Cellular uptake (A) and expression (B) of plasmid DNA. SMC cells were transfected with 2.5 μ g of EMA-labeled or GFP plasmid with PEI25K (N/P=8), with Dosper (Dosper/DNA ratios 1–7.5) or with Dosper–PEI2K (N/P=2.5) combination. The cells were incubated in the transfection solution for 6 h, after which the cells were harvested (EMA) or growth medium was changed and the cells were further incubated for 42 h (GFP). The cells were counted with FACScan ($n=4-11$). When naked DNA was used, cellular uptake (EMA) was $0.51 \pm 0.08\%$ and GFP expression was $0.04 \pm 0.01\%$. *** $P < 0.001$, ** $P < 0.01$.

3.2. Effect of bafilomycin A1

PEI25K-mediated transfection (N/P=8) was sensitive to bafilomycin A1. Bafilomycin decreased the transfection efficiency by 69% at 1 nM concentration. Transfection efficiency was almost completely blocked with 10 nM bafilomycin A1 (Fig. 2). Dosper (Dosper/DNA=7.5) and PEI2K+Dosper were less sensitive to bafilomycin, but transfection efficiency decreased clearly at 1–10 nM bafilomycin. In contrast, Dotap-mediated transfection was not sensitive to bafilomycin A1, even at 30 nM bafilomycin transfection decreased only by 30%. DMSO (at the same concentration than in 30 nM

bafilomycin A1) resulted at least in a 73% transfection (data not shown).

3.3. DNA condensation

The ability of gene carriers to condense DNA was studied by measuring the decrease in the ethidium bromide fluorescence upon its expulsion from DNA. PEI25K was effective in condensing DNA (Fig. 3C). At N/P ratios of 5–10, DNA was condensed to less

Table 1
Ratio of numbers of GFP-expressing cells and EMA-positive cells after exposure to different transfection reagents

Reagent	GFP/EMA
PEI25K/8	0.043
Dosper 1	0.009
Dosper 2.5	0.021
Dosper 5	0.022
Dosper 7.5	0.192
PEI2K/2.5	0.046
PEI2K+Dosper 1	0.008
PEI2K+Dosper 2.5	0.216
PEI2K+Dosper 5	0.224
PEI2K+Dosper 7.5	0.335

GFP/EMA ratio is calculated from the data shown in Fig. 1.

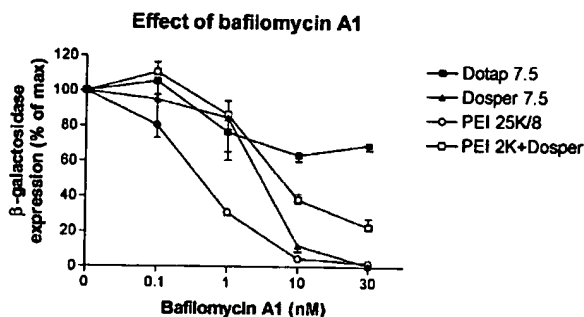


Fig. 2. Effect of bafilomycin A1 on the gene transfection. Serum-free transfection medium was changed to subconfluent SMC cells, and different amounts of bafilomycin A1 were added into wells. After 10 min incubation, transfection solutions were added into wells. After 6 h transfection, growth medium was changed into wells, and cells were incubated for 42 h. The β -galactosidase activity was measured by the ONPG assay. The values are the means of β -galactosidase activity/mg protein \pm S.E. ($n=4-6$).

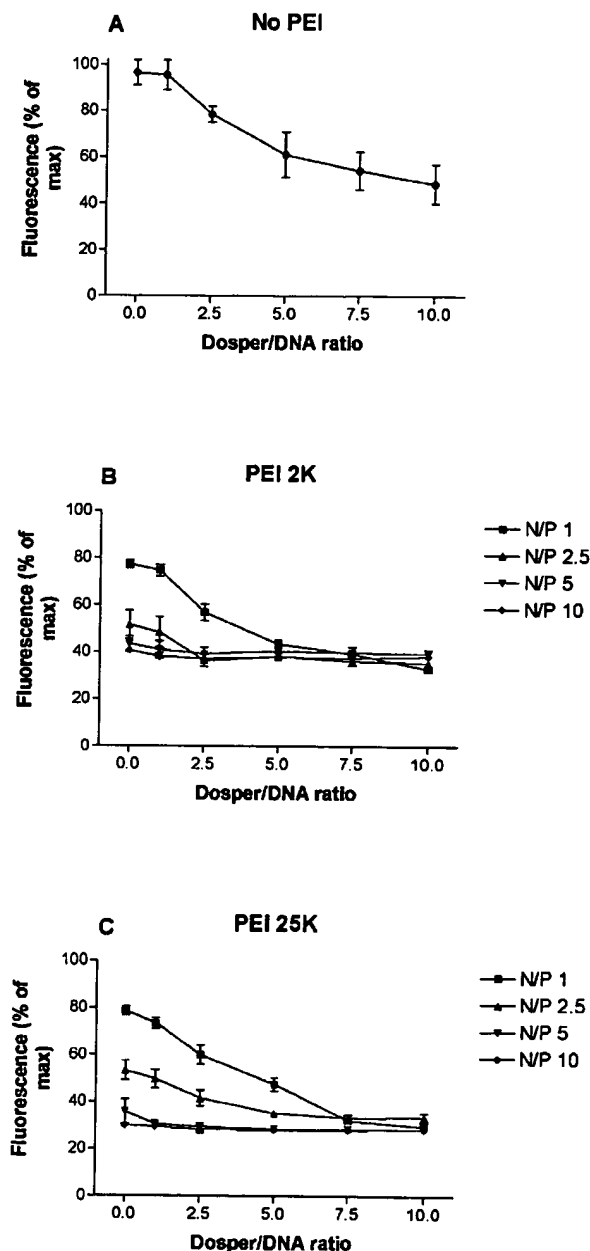


Fig. 3. DNA condensation by Dosper (A), PEI and their combinations. PEI2K (B) or PEI25K (C) was added at different N/P ratios to DNA, and Dosper was then added to wells. The values are percentage of the ethidium bromide fluorescence of plain DNA \pm S.E. ($n=4$).

than 40% from the original, and Dosper did not further condense DNA at these N/P ratios (Fig. 3C). At smaller N/P ratios of 1–2.5, DNA was only condensed to 80 and 55%, respectively, and Dosper could further condense these complexes. At Dosper/DNA ratio of 7.5 condensation was about 30% of the maximum. Also, plain Dosper alone could condense DNA at Dosper/DNA ratios above 1 (Fig. 3A). Fluorescence diminished to about 50% at Dosper–DNA ratios of 7.5–10. PEI2K decreased fluorescence to 40–80% of maximum (Fig. 3B). PEI2K–Dosper and PEI25K–Dosper complexes condensed DNA similarly (Fig. 3B,C). When effective PEI2K N/P ratio of 2.5 [21] was used to pre-condense the DNA prior to addition of Dosper, condensation was increased (Fig. 3B).

3.4. Particle sizing

PEI25K with N/P ratio 8 produced rather small particles with a diameter less than 250 nm (Table 2). Addition of Dosper at the Dosper/DNA ratio of 7.5 did not significantly alter the sizes. With PEI2K, there was much more variability. At the low N/P ratio of 2.5 most of the particles were about 0.5–1.5 μ m in diameter, but there were also particles greater than 3 μ m. Addition of Dosper generated some small particles, but still most particles had diameters 1.5–3 μ m.

3.5. TEM

The TEM results are presented in Fig. 4. Dosper formed small vesicles around 100 nm (Fig. 4A2). PEI2K complexes with DNA appeared as aggregates of various sizes (B1). PEI25K (N/P=2.5) showed aggregates with fine structure of rods (C1) and spheres (C2). Increasing of N/P ratio to 8 condensed the PEI25K aggregates (D1 and D2). Dosper and DNA formed globular complexes of various sizes having striped finestructure (E1 and E2). Adding Dosper to PEI2K–DNA complexes led to rearrangement of the complexes to aggregates (F1) among which remnants of Dosper vesicles can be seen (F2). Adding Dosper to PEI25K–DNA complexes led to rearrangement of the complexes to donuts (G1) and morphologically badly definable aggregates (G2). At the PEI25K–DNA, N/P ratio of 8, mixed with

Table 2
Particle sizes of DNA–complexes with different transfection reagents

Reagent	Size of complexes (μm)							
	<0.1	0.1–0.25	0.25–0.5	0.5–1.5	1.5–3.0	3.0–6.0	6.0–12.0	>12.0
PEI2K (%)	0	0	0	62.8	0	24.1	12.1	1.0
PEI2K+Dosper (%)	0.2	0.6	1.3	5.9	67.1	0	24.7	0
PEI25K (%)	1.4	98.6	0	0	0	0	0	0
PEI25K+Dosper (%)	0.3	99.7	0	0	0	0	0	0

DNA was complexed with PEI25K (N/P=8) or with PEI2K (N/P=2.5). After 10 min incubation, sizes of complexes were determined with Nicomp 380 ZLS zeta potential/particle size for 2×5 min. Dosper (Dosper/DNA=7.5) was then added to complexes, and after 10 min incubation, the sizes of complexes were again determined (n=3). Percentages of complexes in different size classes are presented.

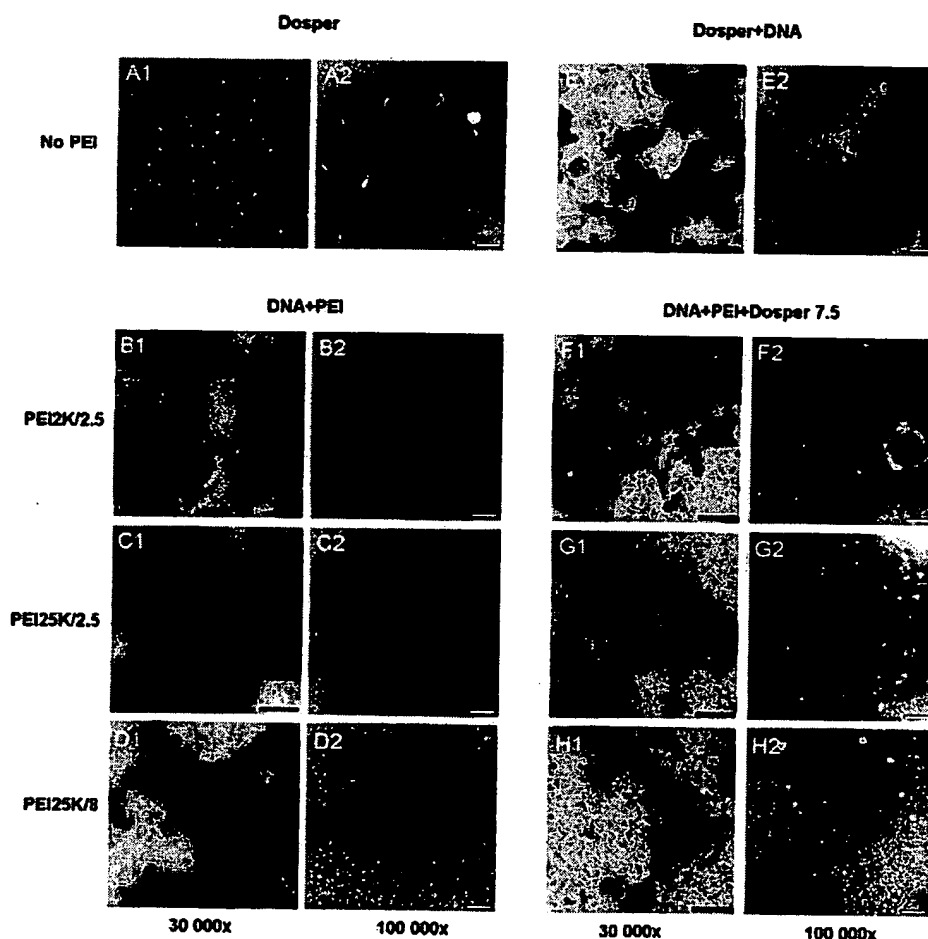


Fig. 4. TEM images of DNA–PEI–Dosper complexes in NaCl–Hepes. Magnifications of 30 000 and 100 000 of each sample are shown as pairs. The eight pairs of figures present Dosper without DNA (A1–A2), PEI2K–DNA (N/P=2.5) (B1–B2), PEI25K–DNA (N/P=2.5) (C1–C2), PEI25K–DNA (N/P=8) (D1–D2), DNA–Dosper (N/P=7.5) (E1–E2) and PEI2K–DNA–Dosper complexes (F–H). Black bar=0.5 μm, white bar=0.1 μm.

Dosper, aggregated complexes (H1) and single donut structures by diameter of about 100 nm and striped fine structures were observed (H2).

4. Discussion

We have shown previously, that combining low-molecular weight PEIs with Dosper liposomes results in a remarkable synergistic enhancement of transfection efficiency [21]. Here we have studied in more detail the mechanisms of this effect.

Ability of different carriers to transfect cells was studied measuring cellular uptake and gene expression. In cellular uptake experiments, more than 70% of the cells took up EMA-labeled plasmid complexed with PEI25K, which is in line with earlier values [23]. PEI2K–DNA without Dosper was poorly taken into the cells. The uptake was only about 2%. It seems evident that the poor efficiency of PEI2K in comparison to PEI25K was due to the low uptake into the cells. The uptake of Dosper–DNA complexes was about 40% and PEI2K in combination did not markedly increase cellular uptake.

Despite the high cellular uptake, only 3% of the cells that took up PEI25K–DNA complexes showed GFP expression (Fig. 1). In the cells transfected with Dosper, the expression was two times higher than with PEI25K and even 15% of the cells transfected with PEI2K–Dosper combinations were expressing GFP (Fig. 1). These results are in agreement with our previous results [21]. Since the PEI–Dosper combination is not toxic to SMC cells [21], toxicity does not explain the increase in GFP expression. Apparently, less than 5% of the cells, which took up DNA with PEI25K, started to produce transgene product (Table 1). In contrast, in Dosper-mediated transfection almost one fifth and with the PEI2K–Dosper combinations one third of the cells with transgene actually produce the protein. Thus, it seems that the basis for synergism is not due to improvements in cellular uptake, but it could rather be explained by the improved intracellular properties. In this experiment, the highest Dosper/DNA ratio was 7.5, which produced the best synergism in our earlier work [21]. PEI25K was not combined with Dosper, since Dosper mainly decreased the

transfection efficiency when combined with PEI25K [21].

It is hypothesized, that the buffering capacity of PEI is one of the crucial properties of the polymer for its high efficiency in the DNA/RNA delivery [12]. This property was studied with bafilomycin A1, a specific inhibitor of vacuolar type H^+ -ATPase. Bafilomycin has been shown to decrease PEI-mediated transfection by inhibiting lysosomal proton pump [11,12]. In our experiments, use of bafilomycin had a similar effect (Fig. 2). The Dosper-mediated transfection was less sensitive to small concentrations of bafilomycin, but at the high bafilomycin concentration of 30 nM, the transfection efficiency was less than 1% compared to control without bafilomycin. This is in agreement to the fact, that Dosper is a polycationic liposome with buffering capacity. Our control lipid, Dotap, is a monovalent cationic lipid with protonation properties independent on the pH at range of 5–7.5 [11]. Bafilomycin caused only moderate changes in the Dotap-mediated transfection so that at 30 nM bafilomycin A1, the transfection efficiency was approximately 70% compared to the control. This does not differ from DMSO control (73%). The endolysosomal proton pump seems to have smaller effect on the transfection efficiency of Dotap than Dosper or PEI25K. Furthermore, the combination of PEI–Dosper is affected less than PEI25K or Dosper alone. Thus, the combination complex may be less dependent on endosomal buffering in its activity.

We studied also the physical properties of the transfection complexes. PEI25K (N/P=8) with or without Dosper generated small particles, approximately 200 nm in diameter when complexed with DNA. These sizes are in agreement with those reported previously [24,25]. However, the sizes of PEI2K–DNA (N/P=2.5) complexes with or without Dosper was more heterogenous and larger. Most of the complexes were 500–1500 nm in diameter, but even larger complexes were found. Also Petersen et al. [26] reported, that small PEIs form huge aggregates. These complexes were much bigger than endosomes, which are approximately 100–200 nm in diameter. The cellular uptake of DNA in these complexes was high (~40%). It is unlikely that such large complexes could enter the cells unaltered. The complexes may bind with the cell surface com-

ponents, such as glycosaminoglycans (GAGs) before they would be taken up into the cells. At this point, the complexes may somehow change. Also, light scattering detects an aggregate as one particle. Based on the TEM pictures and DNA condensation results, the PEI2K–Dosper complexes might be composed of condensed smaller particles that aggregate in solution. It is possible that individual particles are separately endocytosed from the larger aggregates.

The variability of the complexes was seen also with TEM. By the method used [27], it is not possible to get detailed information of the large aggregates, since they collect too much dye to be transparent for the electron beam. Therefore, we will focus on discussing the small particles and the separate complexes. The appearance of the Dosper liposomes (Fig. 4A2) resembled closely to those described before [28]. The Dosper–DNA complexes (Fig. 4E) form striped globules resembling those reported by Labat-Moleur et al. [29] and Birchall et al. [30]. These stripes may be due to a lamellar structure, such as the complexes of cationic lipids and oligonucleotides imaged by freeze fracture TEM [31,32]. The PEI–DNA complexes tended to aggregate at a near neutral N/P and to form more discrete particles at a higher N/P [33]. The same tendency was found in the TEM figures (Fig. 4C,D,G,H). We conclude that mixing of the PEI–DNA complexes with Dosper led to rearrangements of the already polymorphic structures. For example, the striped toroidal polyplexes probably consisted of DNA complexes with PEI and Dosper (Fig. 4H2).

The ability of the carriers to condense DNA was studied with ethidium bromide [18]. Interestingly, the ability of PEI25K and PEI2K to condense DNA was very similar. A further condensation of these PEI–DNA complexes by Dosper was dependent on the N/P ratio of respective PEI. However, the cellular uptake (Fig. 1A) and size (Table 2) of the complexes with PEI25K or PEI2K were quite different. Therefore, it seemed that both PEI initially condense DNA similarly, but the surface properties of these primary particles would be different and therefore the aggregation and cell uptake were also quite different. Since both PEI25K and 2K condense DNA similarly, and Dosper did not further cause any substantial changes, it seemed that the condensation could not explain the synergism.

The clearest result seen in this study was the improved transfection by PEI2K–DNA–Dosper complexes although their cellular uptake was similar with that of Dosper–DNA complexes and lower than that of PEI25K. On the other hand, Dosper facilitated DNA uptake in combination when compared with the PEI2K–DNA complexes. There are several possibilities that may cause the synergism in transfection. Cellular uptake, DNA condensation or complex size do not explain the differences in the transfection efficiency by PEI2K. It is likely that the synergism was based on an altered intracellular behaviour of the complexes. For example, the PEI–DNA–Dosper complexes may interact on the cellular membrane with the extracellular GAGs in different ways. The complexes may shuttle the GAGs into the cells [23] and these associated GAGs may then change the performance of the complexes in the endosomes. Once inside the endosomes, the combination complexes may be less dependent on the acidification of endolysosome, and PEI might have some synergism with Dosper to rupture the endosomal wall. It is suggested, that the rate limiting step in PEI-mediated cell transfection is the transfer of PEI–DNA complexes from the lysosomal compartment to the nucleus [13]. In the cytoplasm DNA may be degraded and its mobility limited [34]. The complexation with PEI could protect the DNA from degradation, or the transport of PEI–DNA complexes to the nucleus could be more effective than that of naked DNA alone [13]. Unlike the cationic liposomes, PEI is also able to target DNA into the nucleus [14]. This property may be more effective with the small PEIs than with larger PEIs.

We conclude that the synergism of PEI–DNA–Dosper complexes in the gene transfection is not due to differences in the cellular uptake, DNA condensation or complex size. It is suggested that PEI2K improves the intracellular kinetics of DNA without affecting cellular uptake, while Dosper is responsible of the cellular uptake of PEI2K–DNA complexes. These two independent actions would lead to a substantial synergism.

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Attenuating the growth of tumors by intratumoral administration of DNA encoding *Pseudomonas* exotoxin via cationic liposomes

Noga Yerushalmi, Ulrich Brinkmann, Elisabeth Brinkmann, Lee Pai, and Ira Pastan

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

A gene therapy approach was taken to inhibit tumor growth by transfecting tumor cells with a plasmid encoding a truncated but active form of *Pseudomonas* exotoxin A (PE), using cationic lipids as the transfection reagent. Cells transfected with this plasmid express PE intracellularly and undergo apoptosis. Transfection was optimized *in vitro* using two cationic lipids, DOGS and DOSPER. A ratio of between 1:4 and 1:10 (wt/wt) was found to be optimal for DOSPER, and the ratio 1:4 was used for the *in vivo* study when a smaller injection volume was desired. Estimating the activity of the PE-encoding plasmid was done both directly, by counting cells *in vitro* after transfection, and by using a cytotoxicity assay, and indirectly, by cotransfecting the plasmid with a plasmid carrying a reporter β -galactosidase gene and observing a reduction in β -galactosidase activity with increasing amounts of the PE-encoding plasmid. The cotransfection method was found to be very sensitive, and showed transfection of cells even with 1–2 ng of the PE-encoding plasmid per 10^5 cells. Complexes of the PE-encoding plasmid together with cationic lipid were injected into tumor xenografts in athymic nude mice. The tumor growth of transfected tumors was attenuated compared with control untreated tumors or tumors transfected with a nontoxin-expressing vector. These results indicate the potential of such a treatment for attenuating solid tumor growth *in vivo*. **Cancer Gene Therapy (2000) 7, 91–96**

Key words: Cationic lipids; transfection; cancer therapy.

In recent years, efforts have been made to use gene therapy as a new approach for cancer therapy.¹ Targeting to tumors is achieved by different delivery systems, including viral and nonviral vectors. Also, different methods have been developed to increase the selectivity by selective targeting or selective gene expression.² Three major approaches are currently being explored for gene therapy for tumors: (a) boosting the immune system to reject the tumor,³ (b) inducing tumor suppressor gene expression,⁴ and (c) eradicating the tumor by delivery of a toxic gene.⁵ In this study, the third approach was investigated using a plasmid encoding a truncated form of *Pseudomonas* exotoxin (PE) via cationic liposomes as the delivery vector.

The mechanism of cell killing by PE is through adenosine diphosphate ribosylation and by inactivation of elongation factor 2 and thus inhibition of protein synthesis.^{6–8} The truncated form of PE encoded by the plasmid used in this study (pULI100) has full activity in terms of protein synthesis inhibition (carried out by domain III of the toxin) and a portion of the toxin that

is responsible for translocating to the cytosol (domain II); however, the binding domain (domain I), which is responsible for binding to α_2 -macroglobulin receptor present on animal cells is, deleted. A cell transfected with this plasmid will express this truncated form of PE and will rapidly undergo apoptosis.⁹

Cationic liposomes are one of the more attractive vectors for gene delivery *in vivo*, because they are not limited by the size of the DNA they carry, and although they are not as efficient as viral vectors, they are less toxic, not immunogenic, and can be produced in larger quantities than viral vectors.^{10–13} A large and growing number of cationic liposomes have been developed in the last several years. In this study, we chose to use cationic lipids with a multivalent cationic head group (spermine), dioctadecylamidoglycyl spermine (DOGS), and 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propyl-amid (DOSPER). The cationic liposomes form a complex with DNA through charge interactions, and the net positively charged complex can interact with cells to produce successful transfections. Cationic liposomes can be administered intravenously.^{14–17} By intravenous injections, the lungs are the organ with the highest yields of transfection. Because of the probable lethality of transfection with PE and the probable damage to non-target tissues, DNA-lipid complexes were injected directly into the tumor. To achieve sufficient cell killing for

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Address correspondence and reprint requests to Ira Pastan, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, 37 Convent Drive, MSC4255, Bethesda, MD 20892-4255.

tumor regression, it is necessary to transfect a large number of cells with high efficiency. In this study, we show a sensitive means of estimating the efficiency of transfection, and the potential of transfection by intratumoral injections *in vivo*.

MATERIALS AND METHODS

Transfection

The transfection of cells *in vitro* was performed with the transfection reagents DOGS (Promega, Madison, Wis) and DOSPER (Boehringer Mannheim, Indianapolis, Ind). DOGS reagent was dissolved in ethanol. Ethanol was evaporated, and the dry lipid film was resuspended in a trace amount of ethanol and RPMI 1640. DNA diluted in RPMI 1640 was added. The mixture was incubated for 20 minutes at room temperature. DOSPER was diluted in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer and 150 mM NaCl (pH 7.4) and was mixed with DNA diluted in the same buffer to different DNA to lipid ratios.

DNA-lipid complexes were added to cells in media with and without sera and incubated for different time periods. After complexes had been removed, 2× full supplemented media was added to the cells.

Assays for expression

An assay for β -galactosidase (β -gal) expression was performed at 24 hours posttransfection by β -gal staining and by using an enzyme-linked immunosorbent assay (ELISA) kit for β -gal quantitation (Boehringer Mannheim). For β -gal staining, cells were washed and fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline. Cells were then covered with a solution of 5-bromo-4-chloro-3-indolyl β -D-galactoside in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ in PBS, and 2% agarose. Cells were incubated overnight at 37°C and checked under a microscope for blue transfected cells. The ELISA kit for β -gal quantitation was used to incubate cell lysates in plates covered with anti- β -gal antibody.

Cell number was measured as follows: cells were washed extensively with PBS and trypsinized, and a fraction was counted in a Coulter counter (Coulter, Hialeah, Fla). Cell lysates were also assayed for protein concentration using Coomassie reagent (Pierce, Rockford, Ill). Cytotoxicity assays were performed using 96-well plates. Cells were plated at a density of 1.5×10^4 cells/well 1 day before the assay. Cells were incubated for 20 hours with different amounts of DNA-lipid complexes and then pulsed with [³H]leucine for 2 hours. After incubation, plates were frozen in dry ice, thawed, and processed in a harvester. Filter mats containing labeled protein were read in a Trilux β -counter (Wallace, Gaithersburg, Md). IC₅₀ was calculated to be the concentration of DNA to give 50% inhibition of protein synthesis.

Tumor injections

A431 cells (3×10^6 cells per mouse) in medium were injected subcutaneously on day 0 into 4- to 6-week-old female athymic nude mice. MCF7ras cells were injected in the same manner, except that cells were suspended in 1:1 vol/vol RPMI 1640 and extracellular matrix (ECM) gel (Sigma, St. Louis, Mo). Each treatment group consisted of five animals. When tumors reached 0.5 cm in diameter, DNA-lipid complexes were injected intratumorally in a volume of 20–40 μ L with 30-gauge

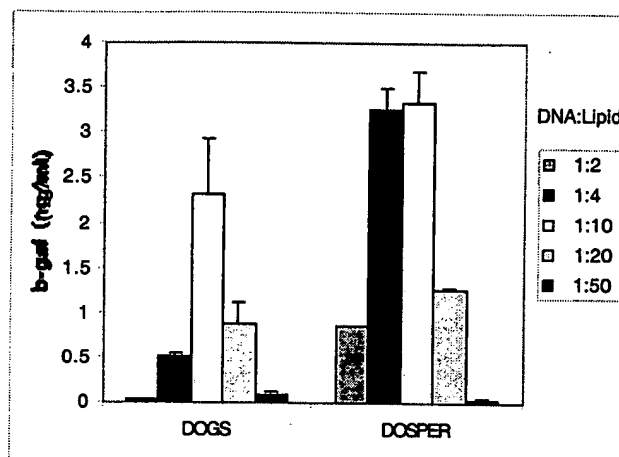


Figure 1. Optimization of the DNA to cationic lipid wt/wt ratio was done in cell culture using the A431 cell line as a model, for both cationic lipid reagents. Transfection was done in duplicate using pELI92 as a reporter plasmid encoding β -gal. β -gal expression was determined using a β -gal ELISA kit.

needles. Tumor measurements were taken with a caliper, and tumor volume was calculated from the following equation: $0.4 \times l \times (w)^2$, with *l* being the tumor length and *w* representing tumor width.

Plasmid construction

pULI100 (plasmid encoding PE35KDEL) and pELI92 (plasmid encoding *Escherichia coli lacZ*) were derived from pCMV β , which contains the cytomegalovirus promoter for expression in eukaryotic cells.¹⁸

RESULTS

Optimization of lipid to DNA ratio

The charge ratio between the DNA and the cationic lipids is important in forming the right complex to yield the best transfection of cells. DNA-lipid complexes with different charge ratios can result in the formation of different particles, which have a large effect on the transfection yield.¹⁹ Different charge ratios were tried ranging from 1:2 wt/wt of DNA to lipid, respectively (1:1.2 charge ratio), up to a ratio of 1:50 wt/wt (1:30 charge ratio). Transfection was measured together with cell viability *in vitro*. Transfection was performed on A431 cells using two cationic lipid reagents, DOGS and DOSPER, and the reporter gene β -gal. Transfection was also optimized for the incubation time and was determined to be 2 hours. β -gal expression was assayed by an ELISA kit and was also checked by β -gal staining (data not shown). Figure 1 shows that for the cationic lipid DOGS, a wt/wt ratio of 1:10 (DNA to DOGS) was optimal under the experimental conditions. The DOSPER reagent gave a better overall transfection efficiency, and a ratio of between 1:4 and 1:10 wt/wt gave maximal transfection yields. For the *in vivo* study, we desired to keep the volume of injection into the tumor as

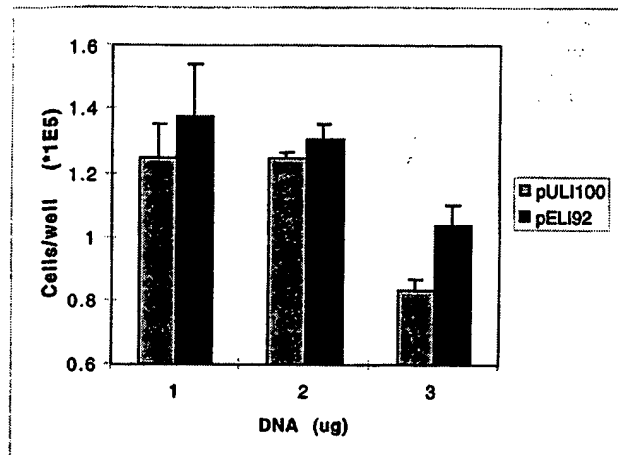


Figure 2. Transfection of MCF7 cells was done in six-well plates using DOSPER as the cationic lipid reagent. Cells were transfected with either pULI100 or pELI92. At 24 hours posttransfection, cells were trypsinized and counted. Each experiment was done in duplicate.

small as possible; therefore a ratio of 1:4 (DNA to DOSPER) was used for the tumor experiments described below.

Assays for transfection yield and activity of PE-encoding plasmid

Cells transfected with the plasmid encoding for PE are expected to produce the toxin and undergo apoptosis. For that reason, a direct way to measure the transfection yield and expression level of the PE-encoding plasmid (pULI100) is to count cells treated with DNA-lipid complexes *in vitro*. Figure 2 shows cells that were transfected with increasing amounts of pULI100. Control cells were transfected with another plasmid encoding β -gal (pELI92). In the figure, we can see that the number of cells per well for the cells transfected with the PE-encoding plasmid decreased with increasing amounts of DNA. The cells transfected with the PE-encoding plasmid underwent apoptosis, which results in a decrease in cell number. The decrease in cell number could also be attributed to lipid toxicity, as we also observed a decrease in cell number for the control group, which was transfected with β -gal-encoding plasmid. However, the decrease in cell number in the cells transfected with the PE-encoding plasmid is more pronounced than the decrease in cell number for the control group. For later experiments, we used subtoxic doses of cationic lipids to avoid the lipid toxicity as a side effect. Although counting total cells is a direct way of measuring PE gene activity, the cells are counted at 24 hours posttransfection; at that time, cells that have not been transfected can overgrow and mask the actual effect of the transfection. For these reasons, we do not consider this assay to be sensitive enough to estimate the transfection yield of our plasmid.

To examine the toxicity of the lipids versus the toxicity of the expressed PE, we performed a cytotoxicity assay.

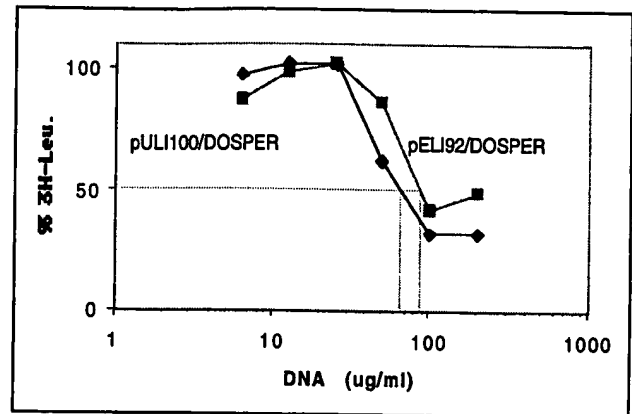


Figure 3. Cytotoxicity assay in A431 cells. Inhibition of protein synthesis was measured in cells treated with pULI100:DOSPER and compared with cells treated with pELI92:DOSPER.

Figure 3 shows a cytotoxicity assay performed on A431 cells that were treated with increasing amounts of DNA (either pULI100 or pELI92) with a similar amount of DOSPER for every concentration (ratio of 1:4 DNA to DOSPER, respectively). The IC_{50} for cells treated with PE-expressing plasmid was 65 μ g/mL, whereas the IC_{50} for the control plasmid was 90 μ g/mL.

Another way to measure the efficiency of transfection with the PE gene is to cotransfect cells with the two plasmids; a PE-encoding plasmid (pULI100) and a β -gal-encoding plasmid (pELI92). Cells transfected with both plasmids undergo apoptosis, resulting in a decrease in β -gal expression. Figure 4 shows the results of cotransfection of two different cell lines, A431 (epidermoid carcinoma) and MCF7 (breast carcinoma) with both the PE-encoding plasmid (pULI100) and the β -gal-encoding plasmid (pELI92). Transfection was done with both cationic lipid reagents. The amount of pELI92 was kept

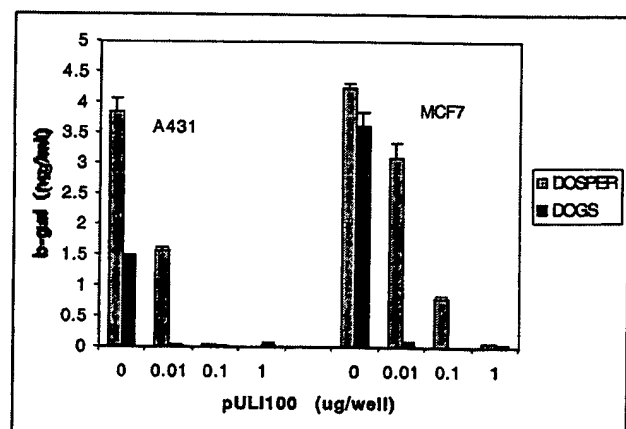


Figure 4. Cotransfection of both pULI100 and pELI92 plasmids was done in two different cell lines, A431 and MCF7, with both cationic lipid reagents. Cotransfection was done using a constant amount of pELI92 (1 μ g/well) and increasing amounts of pULI100. Each experiment was done in duplicate.

constant at 1 $\mu\text{g}/\text{well}$, and it was cotransfected with increasing amounts of pULI100. As seen in Figure 4, even with as little as 0.01 μg of the PE-encoding plasmid (pULI100), we observe a dramatic reduction in the β -gal expression for both cell lines and with both cationic lipid reagents (a $>50\%$ reduction in β -gal expression in A431 cells transfected with DOSPER). Transfection with 0.1 μg of the PE-encoding plasmid resulted in an almost complete eradication of β -gal expression in A431 cells with both transfection reagents. A similar pattern was observed in the MCF7 cell line. It is important to note that in all experimental wells, the lipid amount was subtoxic, and a protein assay showed a similar amount of protein in all wells, suggesting that the decrease in β -gal expression was due to expression of the toxin and was not a side effect of the toxicity. The need for as little as a 1:100 wt/wt ratio of pULI100 and pELI92 to cause cell death in $>50\%$ of β -gal-expressing cells could be due to the fact that cell death is induced with very low expression levels of PE; in contrast, higher expression is needed in the cell to produce a detectable amount of β -gal, and that can only result with transfection of multiple copies of the plasmid into each cell.

Antitumor effect

To determine whether we could use the lethal potential of the PE-encoding plasmid to kill tumor cells *in vivo*, complexes of DNA and DOSPER or DNA and DOGS were injected into tumors composed of A431 cells and MCF7ras cells growing in athymic nude mice. Complexes of DNA with DOGS showed a lower efficiency of transfection, as was expected from our *in vitro* studies (data not shown). Transfection was done by direct injections into the tumors, because most experiments with cationic liposome-DNA complexes that are administered intravenously have been found mainly to transfect cells in the lungs, and to a lesser extent in the spleen, heart, liver, kidneys, and other organs.^{16,19} Because the cationic lipids do not have any specificity for tumor cells, and because transfecting other organs with the PE-encoding plasmid can result in extensive damage to those organs, the intratumoral route of administration was chosen.

Figure 5 shows transfections that were done on A431 tumor-bearing mice. Intratumoral injections were done on days 4, 6, and 8. The figure shows a very slow pattern of growth for the tumors transfected with the PE-encoding plasmid, whereas the control untreated tumors grew quite rapidly ($P = .005$ for day 12). In another experiment, tumors treated in the same manner with a control nontoxin plasmid were found to behave the same as untreated tumors ($P = .75$ for day 18). Because of the rather large SD, experiments were repeated four times to verify the overall result. All experiments had the same profile of attenuated growth in treated tumors compared with controls ($P = .003$ for combined experiments on day 11).

Figure 6 shows a similar experiment done with MCF7ras tumor-bearing mice. This cell line, although

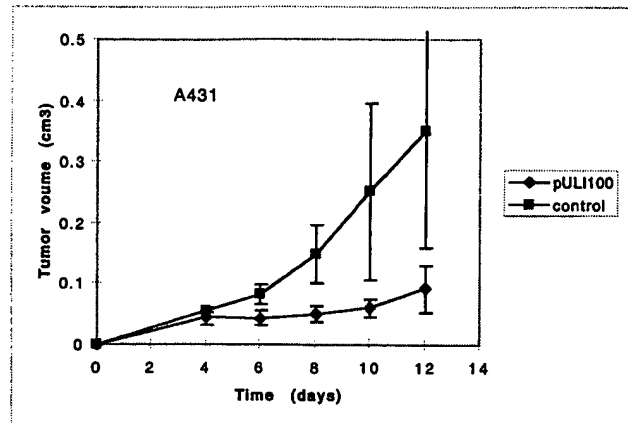


Figure 5. *In vivo* transfection of A431 tumor xenografts in athymic nude mice using pULI100 complexed with DOSPER. Transfection was done with intratumoral injections of DNA-lipid complexes on days 4, 6, and 8. Control tumors were left untreated.

very easily transfected *in vitro*, grows slower as a xenograft in athymic nude mice.²⁰ For that reason, this cell line was transplanted in a suspension of ECM matrigel to enhance the xenograft take.²⁰ As seen in Figures 5 and 6, MCF7ras tumors treated with the PE-encoding plasmid complexed to DOSPER showed not only a slower growth profile but also a decrease in initial tumor size when compared with A431 tumors. The control group in this experiment was treated with the β -gal-encoding plasmid complexed to DOSPER. Control tumors grew faster than those for the treatment group ($P = .0003$ for day 32) although not as fast as A431 control tumors. This slower growth pattern is attributed to the nature of this tumor rather than a result of the transfection with DNA complexed to cationic lipids, as untreated MCF7ras tumors grow as slowly ($P = .38$ on day 15).

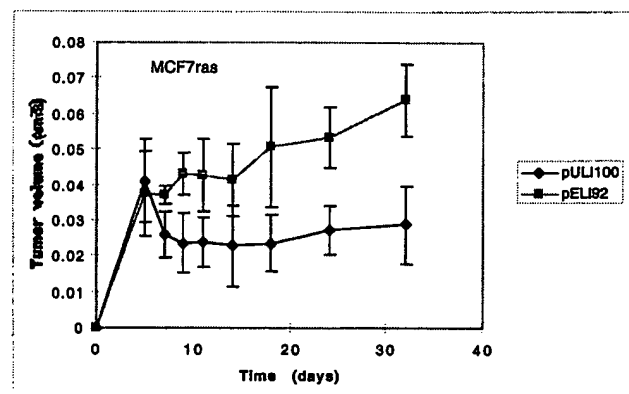


Figure 6. *In vivo* transfection of MCF7ras tumor xenografts in athymic nude mice. MCF7ras cells suspended in ECM gel were injected into mice to form tumors. Transfection was done using pULI100 complexed with DOSPER injected directly into the tumor on days 5, 7, 9, and 11. Control tumors were transfected with pELI92 complexed with DOSPER.

DISCUSSION

The toxic potency of PE was used to reduce the tumor size of tumor xenografts in athymic nude mice by transfecting the tumor cells *in vivo* via cationic liposomes.

We initially examined how different ratios between the plasmid DNA and the cationic lipid reagents affect the transfection yield *in vitro*. This was done to get an idea of the ratio range that should be used for the *in vivo* studies. The transfection level was measured by assay of β -gal expression. We found that the two cationic lipid reagents that were used in this study, DOGS and DOSPER, had different transfection profiles, resulting in a higher transfection yield for DOSPER in all of the cell lines examined. Although a ratio of 1:10 wt/wt DNA to lipid, respectively, was found to give the highest transfection results for both reagents, a ratio of 1:4 was comparable with the transfection yield of the ratio 1:10 in the case of DOSPER. A ratio of 1:4 DNA to lipid, respectively, was later used for our *in vivo* study because it was desired to keep the intratumoral injection volume low.

We then tried to detect cell death within a population of cells transfected with the PE-encoding plasmid (pULI100). We determined the expression of PE as well as the subsequent cell killing following its expression in several ways. The direct way was to measure cell number at 24 hours posttransfection. Although direct, this measurement is sometimes masked with the growth of nontransfected cells. We also compared the cytotoxicity of the PE plasmid complexed to the lipid reagent with the toxicity of a control plasmid complexed to the same lipid reagent to estimate the subtoxic level of the lipid itself; so that we could use subtoxic amounts of DNA to lipid complexes for later experiments. This cytotoxicity assay also showed the specific activity of the expressed PE compared with the toxicity of the lipid reagent itself.

Another way to determine the expression of PE was to detect the reduction of β -gal expression in cells that were cotransfected with both β -gal-encoding and PE-encoding plasmids. Those results showed that transfection of small amounts of plasmid, not detectable with a reporter gene, is sufficient for the expression of enough PE molecules to cause cell death, and therefore a decrease in β -gal expression in cotransfected cells. Most reporter genes are detected with assays that are limited in their sensitivity; therefore, only transfection of multiple copies of plasmid can result in expression that is high enough for detection. Of course PE could also be expressed after multiple plasmid transfection, only to express enough PE to be lethal for the cell, and the plasmid copy number needed is probably much lower than that needed to express β -gal in a detectable amount. This indirect means of measuring *in vitro* the activity of the PE-encoding plasmid by cotransfecting it with another reporter gene is a useful way to estimate the transfection yields of different transfection reagents, because this method is very sensitive and is not limited to the sensitivity of the reporter gene expression assay.

Those results encouraged us to carry out *in vivo* studies even though it is known that transfection *in vivo*

is not always a very efficient procedure. Although cationic liposomes do not yield very high transfection rates compared with viral vectors, they are becoming a transfection reagent of choice for many *in vivo* applications because of their low immunogenicity. In our *in vivo* studies, we showed that due to the high toxicity of PE, the comparatively low transfection yield of cationic lipid reagents was not an obstacle, and the transfection results clearly indicated a decrease in growth rate or a regression of tumor xenografts transfected with the PE-encoding plasmid. In the current studies, only three injections were administered every other day. Presumably, more frequent injections or an increase of the number of injections would have a more profound effect on attenuation of tumor growth.

We have shown that local delivery of a PE-encoding plasmid complexed with cationic liposomes is feasible and results in a significant delay in tumor growth in the nude mouse model. Because local treatment of many epithelioid carcinomas remains suboptimal despite the best conventional therapy, transfection with PE-encoding plasmids warrants further investigation as a treatment option either alone or in combination with other chemotherapy and radiotherapy.

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Brief Communication

A New Efficient Method for Transfection of Neonatal Cardiomyocytes Using Histone H1 in Combination with DOSPER Liposomal Transfection Reagent

Monika Kott,¹ Annekathrin Haberland,² Sergei Zaitsev,² Bernd Buchberger,³ Ingo Morano,¹ and Michael Böttger^{2,*}

¹Max Delbrück Center for Molecular Medicine, 13122 Berlin-Buch, Germany; ²Franz Volhard Clinic at the Max Delbrück Center for Molecular Medicine, 13122 Berlin-Buch, Germany; and ³Roche Diagnostics GmbH, 82372 Penzberg, Germany

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Abstract—Although cationic lipids are successfully used for gene transfer *in vitro*, primary cells such as neonatal cardiomyocytes frequently resist efficient transfection. We show here that the polycationic lipid DOSPER in combination with histone H1 was much more efficient in transfection of neonatal cardiomyocytes than DOSPER alone or other cationic lipids. This has been shown for transfection with the reporter plasmids pSV β -gal and pCMV luc. If viral transfections are not possible, this mild method is an alternative to transfect cardiomyocytes.

INTRODUCTION

Neonatal cardiomyocytes (CM) are terminally differentiated cells with a limited life span in cell culture. They show no potential for proliferation and are refractory to the commonly used nonviral transfection methods. The main problem with lipofection is low efficiency and toxicity at modest and high lipid concentrations (1). Good results were obtained with recombinant herpes (2) and adenoviruses (3) as well as with fusogenic liposomes exploiting the hemagglutinating virus of Japan (HVJ) as a mediator of liposome-cell surface membrane fusion (4).

Searching for alternative transfection methods, we tested a variety of transfection reagents including different cationic lipids, the nuclear protein histone H1 and the calcium phosphate coprecipitation method. H1 histone binds to and condenses transgene DNA (5), confers a nuclear localizing signal to DNA (6) and is transfection-active per se (7, 8). The highest transfection

efficiencies were obtained by combining the amphiphilic lipofectant DOSPER with histone H1. Another advantage of this method is the ability to use smaller concentrations of cationic lipids reducing the toxicity to cell cultures.

MATERIALS AND METHODS

Preparation and Culture of Neonatal Rat Cardiomyocytes. Primary neonatal heart cell cultures were prepared from ventricular tissue of 1–3 day-old Sprague-Dawley rats (Tierzucht Schönwalde GmbH, Schönwalde, Germany) as described previously (9). The removed ventricles were placed into ice-cold calcium ion-free solution A containing 120 mM NaCl, 4.56 mM KCl, 0.44 mM KH_2PO_4 , 0.420 mM Na_2HPO_4 , 25 mM NaHCO_3 and 5.5 mM glucose, pH 7.5, as well as 0.5 mg/ml streptomycin and 5000 IE/ml penicillin G (Biochrom KG, Berlin, Germany). Then minced into pieces of approximately 1 mm² followed by stepwise

disaggregation into single cells in solution A without antibiotics supplemented with 0.12% trypsin (Biochrom KG, Berlin, Germany). The first supernatant with a lot of cell debris and mesenchymal cells was discarded. The supernatant of each digestion step was mixed with ice-cold, heat-inactivated fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and sedimented at $300 \times g$ for 8 min. All pellets were pooled and resuspended in cell growth medium SM20-I supplemented with 10% FCS, 2 mM glutamine, 0.02 mg/ml gentamycin (all Biochrom KG, Berlin, Germany), 0.002 mM fluorodeoxyuridine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to inhibit proliferation of contaminating nonmuscle cells and 2.76 mM hydrocortisone (Sigma).

For enrichment of cardiomyocytes the cell suspension was incubated 90 min at 37°C. During this time most nonmuscle cells (30–40% of the total cell number) attached at the bottom of the culture flask.

The cell number in the supernatant was counted in a Fuchs-Rosenthal chamber. The cardiomyocytes were cultured in 24-well plates (Falcon) or in 25 cm² culture flasks (Costar).

For photographic documentation an inverse phase contrast microscope (Leica DM IRB) and Cannon 500e were used.

Histone H1 and DNA. Nuclear proteins were extracted from calf thymus nuclei with 5% perchloric acid and fractionated by acetone precipitation as described previously. Histone H1 was obtained by FPLC using anionic and cationic ion exchanger columns Mono Q and Mono S (Pharmacia). The fractions 2VAS1 and 2.5VAS1/2 are FPLC-pure H1 (7, 8).

The reporter plasmids pCMV luc and pSV β -gal were grown in *E. coli* DH5 and isolated and purified using standard methods.

Transfection Experiments. Polycationic liposomal transfection reagents DOSPER, DOTAP (Roche Diagnostics GmbH Deutschland), Lipofectamine (Gibco BRL), monocationic liposomal transfection reagents Lipofectin (Gibco BRL), DAC-30 (Eurogentec) and Fugene 6 (Roche Diagnostics GmbH Deutschland) were

tested for transfection efficiency in complex with plasmid DNA and compared with the DNA-calcium phosphate coprecipitation as a standard method. The chemical nature of Fugene 6 has not yet been published. Transfection experiments were performed as recommended by the producers. Briefly, for complexation, 2 μ g plasmid DNA (pCMV luc or pSV β -gal) per well of a 24-well plate (Falcon) were mixed with varying amounts of the following transfection reagents: DOTAP or DOSPER in buffer B (150 mM NaCl, 2.68 mM KCl, 1.18 mM KH₂PO₄, 2.49 mM MgSO₄, 30 mM HEPES, 450 μ M CaCl₂, pH 7.4), Lipofectin or Lipofectamine in OPTIMEM (Gibco BRL), and Fugene 6 in RPMI in buffer C (150 mM NaCl, 10 mM Tris-HCl, pH 7.6) in a final volume of 100 μ l. Samples were shaken about 15 min at room temperature. Calcium phosphate coprecipitation was performed using the Profection kit from Promega. 2.5 μ g DNA were used per well and mixed with 2 M CaCl₂ solution as derived from the protocol of the producer. This solution was mixed with a same amount of 2 \times HBS buffer and gently shaken. Transfection with histone H1 was performed as published previously (7, 8). CaCl₂ was omitted because of the sensitivity of CM to Ca⁺⁺.

Tertiary complexes of DOSPER, H1 and DNA were produced by mixing 2 μ g DNA with 0.5–20 μ g H1 in buffer C as given in RESULTS AND DISCUSSION, then after 5 min gently shaking 3 μ g DOSPER were added (final volume 100 μ l) and gently shaken for further 15 min.

Freshly isolated cardiomyocytes (about 10⁵ cells) were cultivated in 24 well plates on day 1. After 24 h the CM were washed and the cell culture medium SM20-I (Biochrom KG) replaced by fresh medium. Before transfection the culture medium was removed and the cells were washed twice with buffer A. Then the complex solutions were diluted with 900 μ l of buffer B (for DOTAP, DOSPER and H1-DOSPER), Optimem (for Lipofectin and Lipofectamine), or RPMI (for Fugene 6 and Ca phosphate) and

added directly to the cells. After 4 h of incubation at 37°C the transfection mixtures were removed and replaced by normal culture medium SM20-1.

Detection of Transfected Cardiomyocytes. After 24 h the pCMV luc transfected cells were harvested and the luciferase activity measured by the Promega assay system using a Berthold Lumat luminometer. The obtained relative light units (RLU) are related to about 10^4 CM. Data in RLU are the mean of duplicates of at least 3 separated experiments with the standard error shown.

The β -galactosidase activity was measured 2.5 days after transfection with the pSV β -gal plasmid. Cells were washed twice with buffer A and fixed with 2.0% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature, rinsed twice with buffer A and stained with X-gal (Gibco) solution as usual.

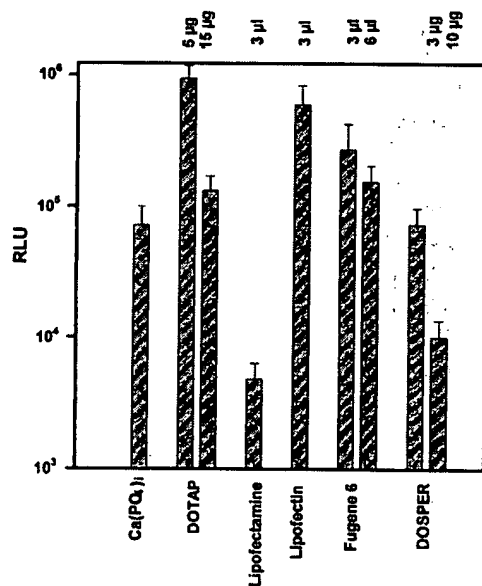


Fig. 1. Luciferase activity in terms of RLU (relative light units) of a number of mono- and polycationic transfection reagents, Eugene 6 and of the DNA-calcium phosphate coprecipitation on neonatal cardiomyocytes of the rat. 2 μ g pCMV luc DNA were complexed with the given amounts of transfectants and added to 10^5 CM. The values are means \pm SE, at least 3 independent experiments.

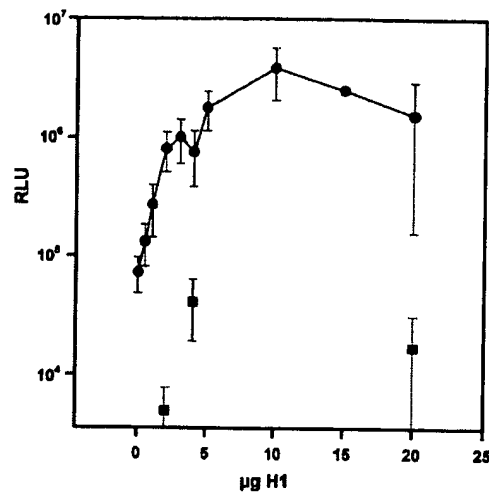


Fig. 2. Effect of H1 concentration on the luciferase activity of ternary H1-complexed DNA/DOSPER complexes on neonatal cardiomyocytes. 2 μ g pCMV luc were complexed with the H1 amounts given and packaged with 2 μ g DOSPER into ternary complexes (●); H1 histone was complexed with DNA in the absence of DOSPER (■). 10^5 cells per well. The values are means \pm SE, at least 3 independent experiments.

RESULTS AND DISCUSSION

Neonatal and adult CM are known to be refractory to nonviral methods of gene transfer. In this study we investigated possibilities to improve the transfection efficiency of commercial lipid formulations (lipofection) on cultured CM by interacting histone H1-complexed DNA with the cationic lipid (10–12). As an exemplary lipid reagent we used the polycationic reagent DOSPER carrying 4 positively charged amino groups.

Figure 1 shows the transfection efficiencies in terms of RLU of a variety of different lipid reagents and of the calcium phosphate coprecipitation method as a control for the combination experiments. The lipids were used according to the recommendations of the producers, not particularly optimized for CM. Best results were obtained with DOTAP and Lipofectin. Lipofectamine was nearly negative (the blank value with naked DNA is about 2000 RLU). Whereas the lipid reagents were not toxic for CM in the

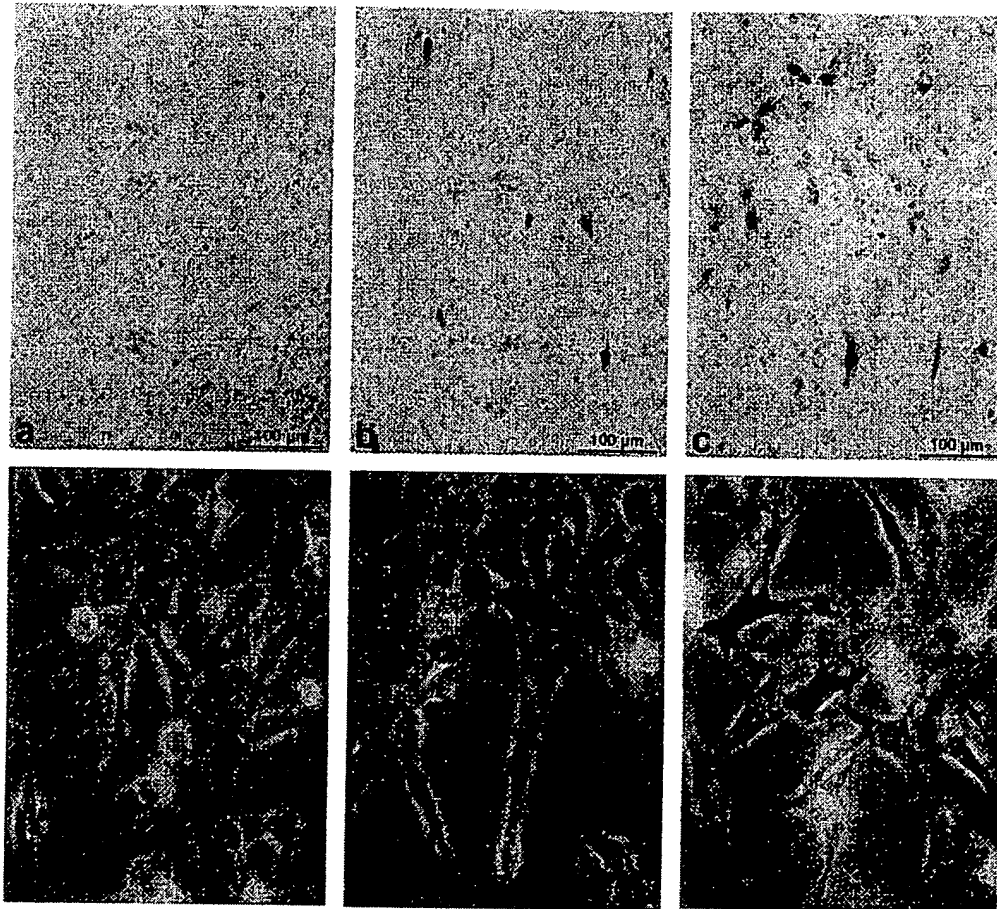


Fig. 3. β -gal expression on neonatal cardiomyocytes (10^5 cells per well)). a,d) DNA controls (2 μ g), b,e) neonatal cardiomyocytes (CM) transfected by pSV β gal-DOSPER complexes (2 μ g DNA + 3 μ g DOSPER), c,f) CM transfected by pSV β -gal-DOSPER-H1 ternary complexes (2 μ g DNA + 3 μ g DOSPER + 10 μ g H1). Fig. 3 a, b and c show the distribution of transfected CM by bright field microscopy, d, e and f in reversed phase contrast.

concentrations used, the calcium phosphate coprecipitation method damaged the CM extremely and was not qualified for this type of cells. After transfection with lipids and incubation for 4 h, the CM proved to be morphologically healthy and their spontaneous pulsation rate remained at 160 imp/min.

Figure 2 shows the transfection efficiency in RLU of the H1-complexed DNA packaged with DOSPER into tertiary complexes as a function of the H1 input. We varied the amount of H1 as shown and selected a firm amount of

DOSPER (3 μ g). There is a maximum of RLU at 10 μ g H1 exceeding the value of DOSPER without H1 by a factor of more than 50. RLU values of selected H1-DNA complexes are also given in Fig. 2. They are smaller than that of DOSPER without H1. This demonstrates that it is possible to enhance the transfection efficiency on CM by a combination of DOSPER and H1. Clearly, more optimization would be necessary in order to obtain maximum transfection.

Figure 3 shows β -galactosidase expression in transfected CM cultured in a 24-well plate.

CM transfected at 4 days postseeding with pSV β -gal and 3 μ g DOSPER/10 μ g H1 (c) are compared with DOSPER (3 μ g) transfected cells (b) and DNA controls (a). A transfection rate of about 5% for DOSPER in the presence of H1 and <0.1% in the absence of H1 was obtained.

Tertiary complexes of cationic lipids, together with a DNA-binding protein like histone H1 and DNA enhance transfection efficiency as has been recently described (10–12). These complexes exploit the transfection-mediating properties of both constituents, lipids and histone, resulting in synergistic effects on transfection. In the absence of calcium in the culture medium the transfection efficiency of H1 alone is low (7, 8). Histone H1 adds the properties of mediating nuclear import although it does not have a defined nuclear localizing sequence (6), DNase protection (10) as well as DNA condensation (5, 13) to the DNA packaging properties of lipids (14). The combination of DOSPER and H1 has not been described. It proved to be suitable to transfect neonatal CM with high efficiency. It is superior to lipofectin, DOTAP and Eugene 6. Higher efficiencies were only obtained with herpes (2) and adenoviruses (3) and fusogenic HVJ liposomes (4).

Transfection results on embryonic CM obtained by other authors (1, 15) are not clear. Whereas lipofection resulted in similar efficiencies as described here for neonatal CM (1), more than 40% of β -gal positive cells were obtained using the calcium phosphate coprecipitation method (15). However, it should be noted that embryonic CM could be transfected more easily with high efficiency because of their replication-competence.

ACKNOWLEDGMENTS

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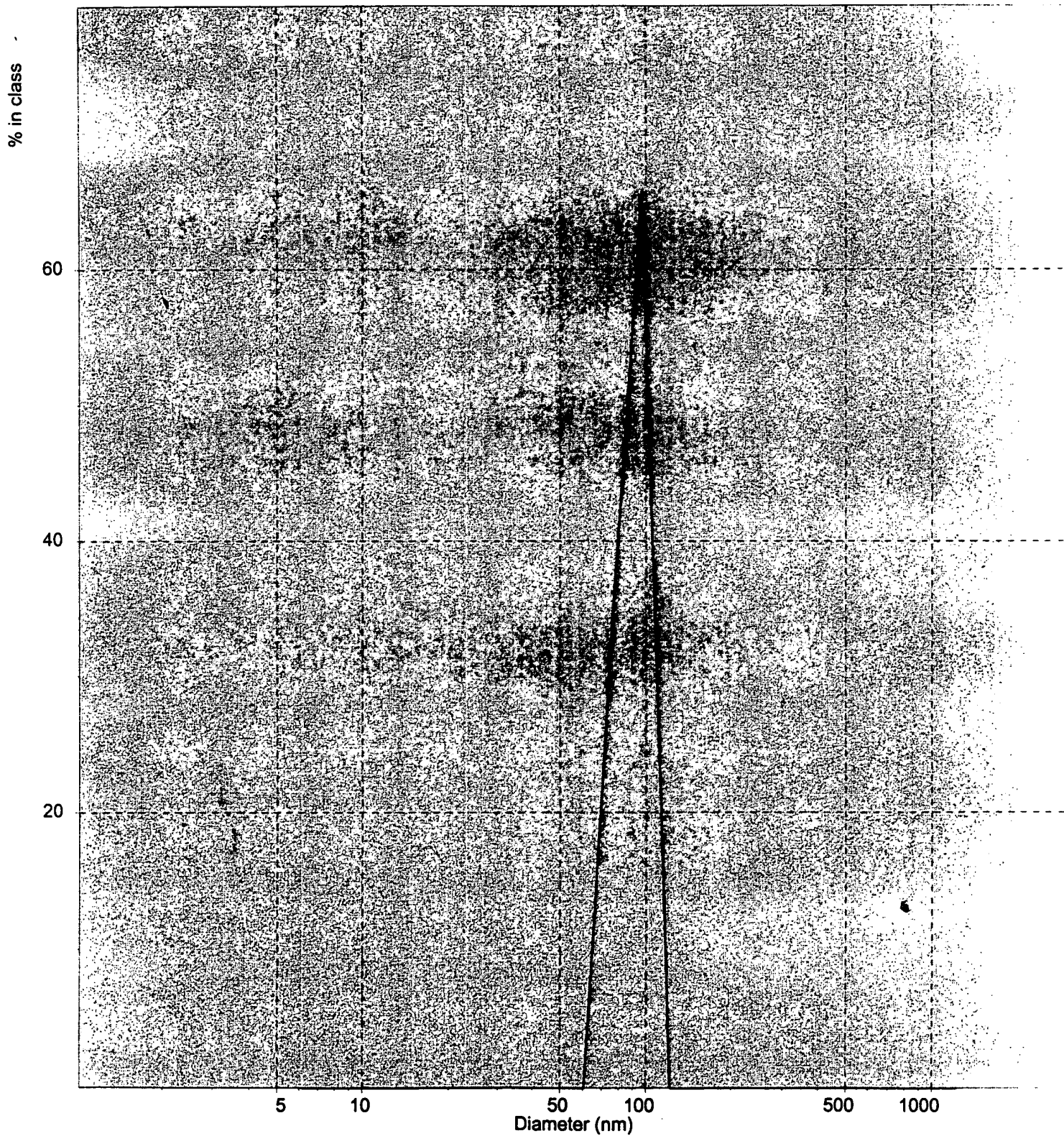
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pCI-HA10
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File data from Live size Record 2
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